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The POU Transcription Factor Oct-1 Represses Virus-Induced Interferon A Gene Expression

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Alpha interferon (IFN-α) and IFN-β are able to interfere with viral infection. They exert a vast array of biologic functions, including growth arrest, cell differentiation, and immune system regulation. This regulation extends from innate immunity to cellular and humoral adaptive immune responses. A strict control of expression is needed to prevent detrimental effects of unregulated IFN. Multiple IFN-A subtypes are coordinately induced in human and mouse cells infected by virus and exhibit differences in expression of their individual mRNAs. We demonstrated that the weakly expressed IFN-A11 gene is negatively regulated after viral infection, due to a distal negative regulatory element, binding homeoprotein pituitary homeobox 1 (Pitx1). Here we show that the POU protein Oct-1 binds in vitro and in vivo to the IFN-A11 promoter and represses IFN-A expression upon interferon regulatory factor overexpression. Furthermore, we show that Oct-1-deficient MEFs exhibit increased in vivo IFN-A gene expression and increased antiviral activity. Finally, the IFN-A expression pattern is modified in Oct-1-deficient MEFs. The broad representation of effective and potent octamer-like sequences within IFN-A promoters suggests an important role for Oct-1 in IFN-A regulation.

Alpha interferon (IFN-α) and IFN-β are able to interfere with viral infection. They exert a vast array of biologic functions, including growth arrest, cell differentiation, and immune system regulation (for reviews, see references 28 and 51). This regulation extends from innate immunity to cellular and humoral adaptive immune responses. A strict control of expression is needed to prevent detrimental effects of unregulated IFN. IFN transcription is coordinately induced in human and mouse cells infected by virus. Multiple IFN-A subtypes exhibit differences in expression of their individual mRNAs.

IFN-A transcription is regulated by a number of different activators and repressors. Among these factors, the interferon regulatory factors (IRFs) play an important role in the stimulation of cellular antiviral defense mechanisms in different cell types. IRFs regulate transcription by interacting with gene promoter sequences. Until now, repressors involved in negative regulation of the IFN-A genes have not been well characterized (for a review, see reference 29). We have shown that in addition to substitutions in proximal virus responsive element A (VRE-A) (2), the low expression levels of the IFN-A11 and IFN-A5 genes after virus induction are also due to the presence of a distal negative regulatory element (DNRE) of 20 bp, which is delimited upstream of VRE-A (20, 25, 26). The analysis of the DNRE responsible for the virus-induced transcription repression of some IFN-A promoters led us to study the homeodomain transcription factor Pitx1 (25). Upon virus induction, Pitx1 negatively regulates the transcription of DNRE-containing IFN-A11 and IFN-A5 promoters (20, 25). We have recently shown that Pitx1 inhibits the IRF-3 and IRF-7 transcription activation of the IFN-A11 and IFN-A5 promoters and interacts physically with IRF-3 and IRF-7 (20).

Here we show that the POU protein Oct-1 binds in vitro to the DNRE and in vivo to the endogenous IFN-A11 promoter in mock-induced and induced cells. Furthermore, Oct-1 represses IFN-A11 expression upon IRF overexpression. Moreover, we show that Oct-1-deficient MEFs exhibit increased in vivo IFN-A gene expression and increased antiviral activity. Finally, the IFN-A expression pattern is modified in Oct-1-deficient MEFs. The broad representation of effective and potent octamer-like sequences within IFN-A promoters suggests an important role for Oct-1 in IFN-A regulation. We suggest this could have implications in IFN-α-based combinatorial therapies.

MATERIALS AND METHODS

DNA transfection, viral induction, and transfection assays. Murine L929 cells were transfected by the standard calcium phosphate precipitation method as previously described (26). Newcastle disease virus (NDV) induction was carried out 24 h later. The mock-induced cells were set up as described above except that no NDV was added. Cells were harvested 24 h postinduction, and cytoplasm extracts were prepared. Luciferase activity was measured in cell lysates by using commercial reagents (Promega). Transfection efficiency was determined by a β-galactosidase activity assay with a chemiluminescent kit (Tropix). In each experiment, a given construction was transfected in duplicate and two different clones of each construction were tested. Each experiment was realized at least five times. The means and standard errors for transcription activity determined by at least five separate experiments are shown.

Plasmid constructions. The IFN-A11 and −330 IFN-B promoters already described (25) were cloned into the pBL-Luc vector. Mutant promoters were made by double PCR as previously described and cloned into pBL-Luc (25). The pBL-Luc vector was derived from the pBL-CAT3 reporter by replacing the CAT gene by the luciferase fragment. All constructions were checked by nucleotide sequencing on a double-stranded DNA template. IRF-3, a gift from J. Hiscott,

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was subcloned into the pcDNA plasmid (Invitrogen), and the pcDNA-IRF-7A expression vector was a gift from J. S. Pagano. Oct-1 and Pitx1 cDNAs, gifts from J. Drouin, were subcloned, respectively, into pcDNA3.1(+) and pRc-CMV2 (Invitrogen).

Chromatographic fractionation of Pitx1 partner binding activity. Nuclear extracts were prepared from L929 cells and fractionated successively by using the following chromatographic matrices: heparin ceramide (HEP) (Amersham Biosciences), sulfopropyl (SP) (Amersham Biosciences), and hydroxyapatite (HA) (prepared in the laboratory). Elution buffers are as follows: for HEP, 50 mM Tris-HCl (pH 8) with 100 (buffer a), 200 (buffer b), or 400 (buffer c) mM NaCl; for SP, 10 mM Tris-HCl (pH 6) with 100, 200, 400, or 800 mM NaCl (buffers d to g, respectively); and for HA, 10, 50, 100, 200, 400, or 800 mM phosphate buffer (pH 6.6) (buffers h to m, respectively). Binding activities of the different fractions eluted were tested for the presence of the Pitx1 partner by electrophoretic mobility shift assay (EMSA) using the DNRE probe as described below. The positive fraction purified successively through the three chromatographic columns was loaded on denaturing polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). The gel containing the fractionated proteins was cut transversely, and proteins from each piece were eluted as described previously (35). Binding activities of eluates were assayed by EMSA with the DNRE probe.

EMSA. Nuclear extracts from L929 cells were prepared as previously described (20) in the presence of protease inhibitors. EMSAs were performed as described previously (20), with some modifications in order to optimize Pitx1 partner binding. Five micrograms of nuclear extracts was incubated with 1 μg poly(dG-dC) · poly(dG-dC) in the presence of 100 ng of sonicated salmon sperm DNA (Amersham Biosciences) for 15 min on ice. After incubation, the mixture was added to the binding buffer containing 10 fmol of ^{32}P-end-labeled probe (50,000 cpm, 0.1 ng) either with or without specific competitor oligonucleotides in a final volume of 20 μl, and the incubation was carried out for a further 30 min at room temperature. For supershift experiments, nuclear extracts were incubated on ice with the specified antibody, anti-Pit1 or anti-Oct-1 (Santa Cruz Biotechnology), for 1 h at 4°C prior to the addition of the labeled oligonucleotide. The samples were subjected to electrophoresis on 6% nondenaturating Tris-borate-EDTA polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography or to PhosphorImager (Molecular Dynamics). The following chemically synthesized double-stranded oligonucleotides (MWG Biotech) were used as probes or competitor (unrelated nucleotides are written in lowercase, and nucleotide mutations are underlined): DNRE (5′-cagATTTAAGTCTAATTTAAAGTcgt-3′), T3 (5′-cagATTTACTACTAATTTAAAGTcgt-3′), T5 (5′-cagATTTAAGTCTACGGTAAAGTcgt-3′), T8 (5′-cagATTTACTACTACGGTAAAGTcgt-3′), T9 (5′-cagATTTACAGCTACTGTAAAGTcgt-3′), Oct-1 gel shift oligonucleotide (OCT) (5′-tgtCGAATGCAAATCACTAgaa-3′), mutant of OCT probe (mOCT) (5′-tgtCGAATGCAAGCCACTAgaa-3′), IFN-A7 probe (5′-ctgCATATAGTGAAATTTAATGAcag-3′), and IFN-A5 probe (5′-ctgAAAATTCAAATTAGGAAGAcag-3′).

In vitro-transcribed and -translated reticulocyte lysates were treated as nuclear extracts. Oct-1 and Pitx1 proteins were produced using the TNT-coupled transcription-translation rabbit reticulocyte lysate system (Promega) with T7 RNA polymerase promoters (pRc-CMV2 and pcDNA3.1). Plasmids pRc-CMV2-Pitx1 and pcDNA3.1(+)-Oct-1 were used for translation of murine Pitx1 and Oct-1 proteins, respectively.

RT-PCR. Wild-type and Oct-1-deficient MEFs were used. Total RNA (5 μg) was subjected to reverse transcriptase PCR (RT-PCR) by standard methods using a poly(dT) primer for reverse transcription and consensus conserved primers annealing with all IFN-A subtypes for PCR amplification: coding, 5′-ATGGCTAGRCTCTGTGCTTTCCT-3′; noncoding, 5′-AGGGCTCTCCAGAYTTCTGCTGCTCTG-3′. The level of IFN mRNA was quantified by using serial dilution RT-PCR as previously described (25). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as controls: sense (5′-TGAAGGTCGGAGTCAACGGATTTGGTCGTATTG-3′) and antisense (5′-ATGTGGGCCATGAGGTCCACCACCCTGTT-3′) oligonucleotides. Thirty cycles of amplification were performed. IFN-A amplified by RT-PCR was purified and cloned with the pGEM-T vector system I (Promega) and subsequently sequenced (ABI Prism; Applied Biosystems).

Protein-DNA immunoprecipitation. L929 cells were treated as described by the manufacturer (Upstate). Briefly, cells were fixed with 1% formaldehyde for 10 min, washed twice, scrapped, and collected by centrifugation. Cell pellet (2×10^7) was resuspended in 0.2 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1] with 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin A/ml, and 1 μg of aprotinin/ml) for 10 min in ice. Cell lysate was sonicated and cleared by centrifugation. Sonicated cell supernatant was diluted 10-fold in chromatin immunoprecipitation dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl with protease inhibitors). Aliquots were used as input DNA control. Diluted cell supernatant was precleared twice with 40 μl of salmon sperm DNA-protein A agarose for 30 min. The supernatant fraction was then incubated with 8 μg of anti-Oct-1, anti-IRF3, or anti-IRF7 immunoglobulin G (IgG) (Santa Cruz Biotechnology) or anti-Pitx1 antibody (prepared in the laboratory) or, as a negative control, anti-β-tubulin IgG (Sigma) antibody overnight at 4°C. Immune complexes were collected on protein A beads preadsorbed with sonicated single-stranded DNA. Beads were washed sequentially in low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]), and twice with Tris-EDTA buffer. The protein-DNA complex was then eluted from the antibody with 1% SDS and 0.1 M $NaHCO_3$. Protein-DNA cross-links were reversed in 0.2 M NaCl by being heated at 65°C for 4 h. The DNA sample was recovered by phenol-chloroform extraction and ethanol precipitation. PCR analysis was performed with primers specific for the IFN-A11 promoter (−457 to −113) or primers specific for pericentromeric gamma-satellite DNA, as described previously (44).

Protein-protein interaction assays. Protein-protein interaction assays were performed using MBP fusion proteins coupled to amylose-Sepharose beads (New England Biolabs) and 5 to 10 μl of in vitro-translated ^{35}S-labeled protein incubated in the presence of binding buffer (200 mM NaCl, 20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.25% bovine serum albumin) for 2 h at 4°C with agitation and then centrifuged at 3,000 rpm at room temperature. Beads were washed five times in binding buffer at room temperature; the protein complexes were released after boiling in Laëmmli buffer and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Labeled proteins were visualized by autoradiography. For binding assays with nuclear extracts, 250 μg of L929 nuclear extracts induced by NDV for Oct-1 and IRF-3 or 250 μg of HeLa S3 expressing IRF-7 and induced by NDV was incubated with MBP fusion proteins bound on beads for 4 h at 4°C with agitation in 250 μl of binding buffer (20 mM HEPES KOH [pH 7.9], 50 mM KCl, 1 μM $ZnSO_4$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.01% Igepal CA-630 [Sigma], 20% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The resulting binding complexes were washed in the same binding buffer five times, and the bound proteins were separated by SDS-PAGE. Proteins were transferred on a Hybond polyvinylidene difluoride membrane and subjected to immunoblotting. Anti-Oct-1, anti-IRF3, and anti-IRF7 antibodies (Santa Cruz Biotechnology) were used. Western blot analyses were done using chemiluminescence as described by the manufacturer (Amersham Biosciences). For coimmunoprecipitation experiments, each assay was carried out in 460 μl of precipitation buffer (20 mM HEPES KOH [pH 7.9], 50 mM KCl, 0.1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5% glycerol) containing 200 μg of L929 nuclear extracts induced by NDV for Oct-1 and IRF-3 or 100 μg of HeLa S3 expressing IRF-7 and induced by NDV. Anti-Oct-1, anti-IRF3, and anti-IRF7 antibodies (Santa Cruz Biotechnology) were used for coimmunoprecipitation. An unrelated polyclonal IgG (INC Technologies) was used as the negative control. After overnight incubation on a wheel at 4°C, 40 μl of protein A-Sepharose (Amersham Biosciences) was added for 1 h at 4°C. The mixture was then centrifuged, and the pellets were washed four times in the same buffer at 4°C. Pitx1 was revealed by Western blotting using anti-Pitx1 antibody.

Antiviral activity assays. Wild-type MEFs or Oct-1-deficient MEFs were incubated with NDV for 1 h. Supernatants were isolated 8 h after virus removal. Monolayer L929 cells in 96-well plates were incubated with serial dilution of supernatants for 24 h before vesicular stomatitis virus (VSV) infection. VSV was added directly to the culture medium. Cells were stained with crystal violet as the vital dye 24 h after infection. The results were quantified using a Titertek Multiskan instrument with a 595-nm filter. One hundred percent cell viability corresponds to the intensity of staining measured in noninfected cells.

RESULTS

The DNRE contains a binding site for the homeodomain transcription factor Pitx1 (20, 25). Pitx1 was shown to be involved in repression of IFN-A11 and IFN-A5 gene transcription. However, Pitx1 by itself cannot account for repression of murine IFN-A11 gene expression. Indeed, we tested whether sequences around the Pitx1 binding site within the DNRE may contribute to transcription repression of VRE-A11, which binds IRF-3 and IRF-7 (Fig. 1A). The DNRE was scanned for

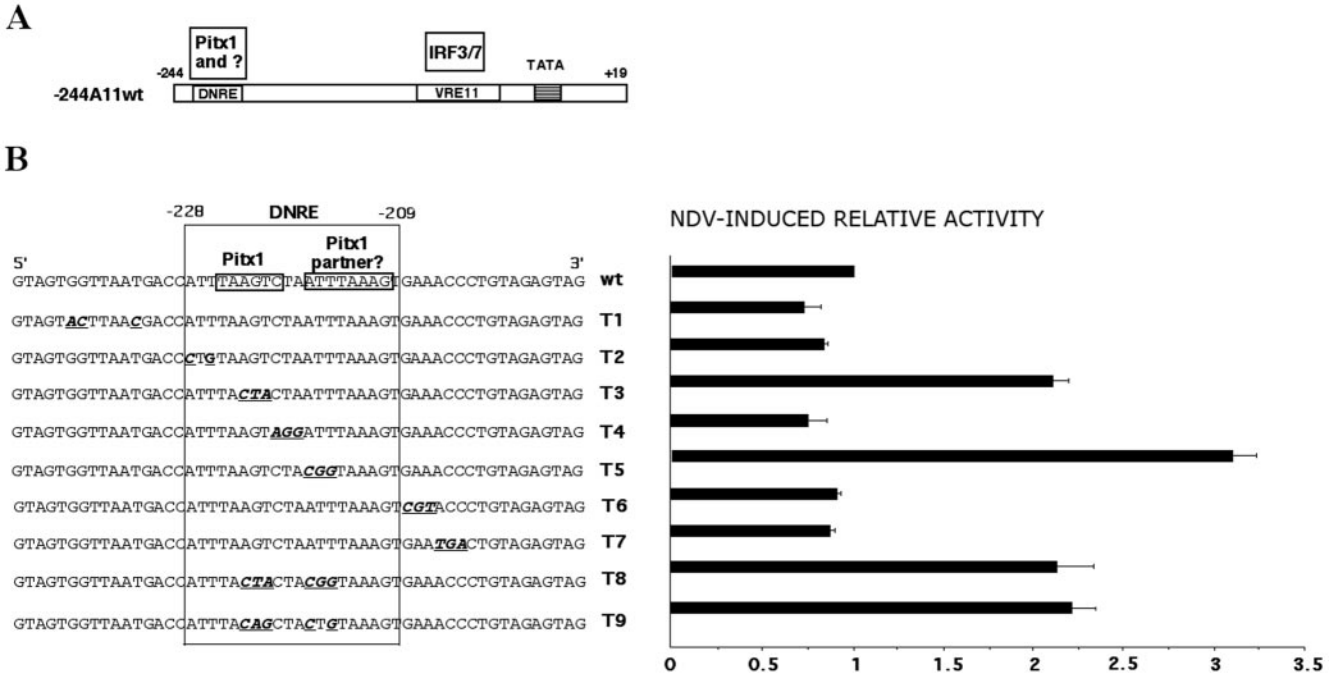

FIG. 1. Pitx1 and Pitx1 partner binding sites are required for repression of IFN-A11 transcription after virus induction. (A) Regulatory mechanism of IFN-A11 promoter from bp −244 to +19 (−244A11wt). The negative DNRE and positive VRE-A11 regulatory elements are shown as open boxes. The DNRE contains a binding site for Pitx1 (25) and for another DNA binding protein (present work), and VRE-A11 is a binding site for IRF-3 and IRF-7 activators. Distances from the transcription start site are indicated in base pairs. The TATA box is depicted as a shaded box. (B) Transcriptional activity of reporter plasmids dependent on the wild type or mutant of the IFN-A11 promoter containing the DNRE in NDV-induced L929 cells. Cells were transiently transfected with the −244A11wt promoter or with the indicated mutant. The DNA sequence of the intact DNRE (wt) is shown above that for each mutant (nucleotide mutations are italic, bold, and underlined). Sequences important for reporter activity are boxed and identified. All values are expressed relative to that of −244A11wt activity in L929 cells induced by the virus. In each experiment, a given construction was transfected in duplicate and two different clones of each construction were tested. Each experiment was realized at least five times. The means and standard errors for transcription activities determined by at least five separate experiments are shown. (C) Sequence alignment of the Pitx1 partner binding site with a POU family protein binding site (57).

active sequences by using a set of mutations and assessed for activity in the context of the IFN-A11 promoter in NDV-induced L929 cells (Fig. 1B). As expected, mutants T3, T8, and T9, which destroyed the Pitx1 binding site, showed increased activities compared to that of the intact DNRE. In addition, mutant T5 was also devoid of negative activity, suggesting another transcription factor binding site. As these two sites are contiguous, we consider this transcription factor a potent Pitx1 partner. The sequence altered by mutation T5 contains the motif ATTTAAAG, which is almost identical to the POU family homeodomain binding site ATTTGCAT. Thus, a POU family homeodomain protein could bind the DNRE (Fig. 1C).

The nuclear Pitx1 partner binds to the DNRE. We used an EMSA-based strategy to identify Pitx1 partner binding to the DNRE. The Pitx1 partner binds in vitro to the wild-type DNRE and to the T3 mutant of the Pitx1 binding site (Fig. 2A, lanes 1 and 2) but not to the T5, T8, and T9 mutant probes (Fig. 2A, lanes 3 to 5). Together with the results of the transfection assays, this indicates that the repression of IFN-A11 gene expression correlates with efficient Pitx1 partner binding to the DNRE (Fig. 1B).

Chromatographic fractionation of Pitx1 partner binding activity led to Oct-1 identification. In order to identify the components of the Pitx1 partner, nuclear extracts were prepared from L929 cells and fractionated by using the following chromatographic matrices: HEP, SP cationic ion exchange, and HA (see also Materials and Methods). Binding activities of the different fractions were tested for the presence of the Pitx1 partner protein by EMSA using the DNRE probe (Fig. 2B). Nuclear extracts were used as a positive control with the DNRE probe (Fig. 2B, lane 1). The fraction positive for the Pitx1 partner was eluted from the HEP matrix with elution buffer b (Fig. 2B, lane 3). The positive fraction was subsequently fractionated on the SP matrix. The positive fraction was essentially eluted in buffer e (Fig. 2B, lane 6) and subsequently fractionated on the HA matrix (Fig. 2B, lanes 9 to 14).

In order to determine the approximate molecular mass of the Pitx1 partner, the positive fraction eluted in buffer k from

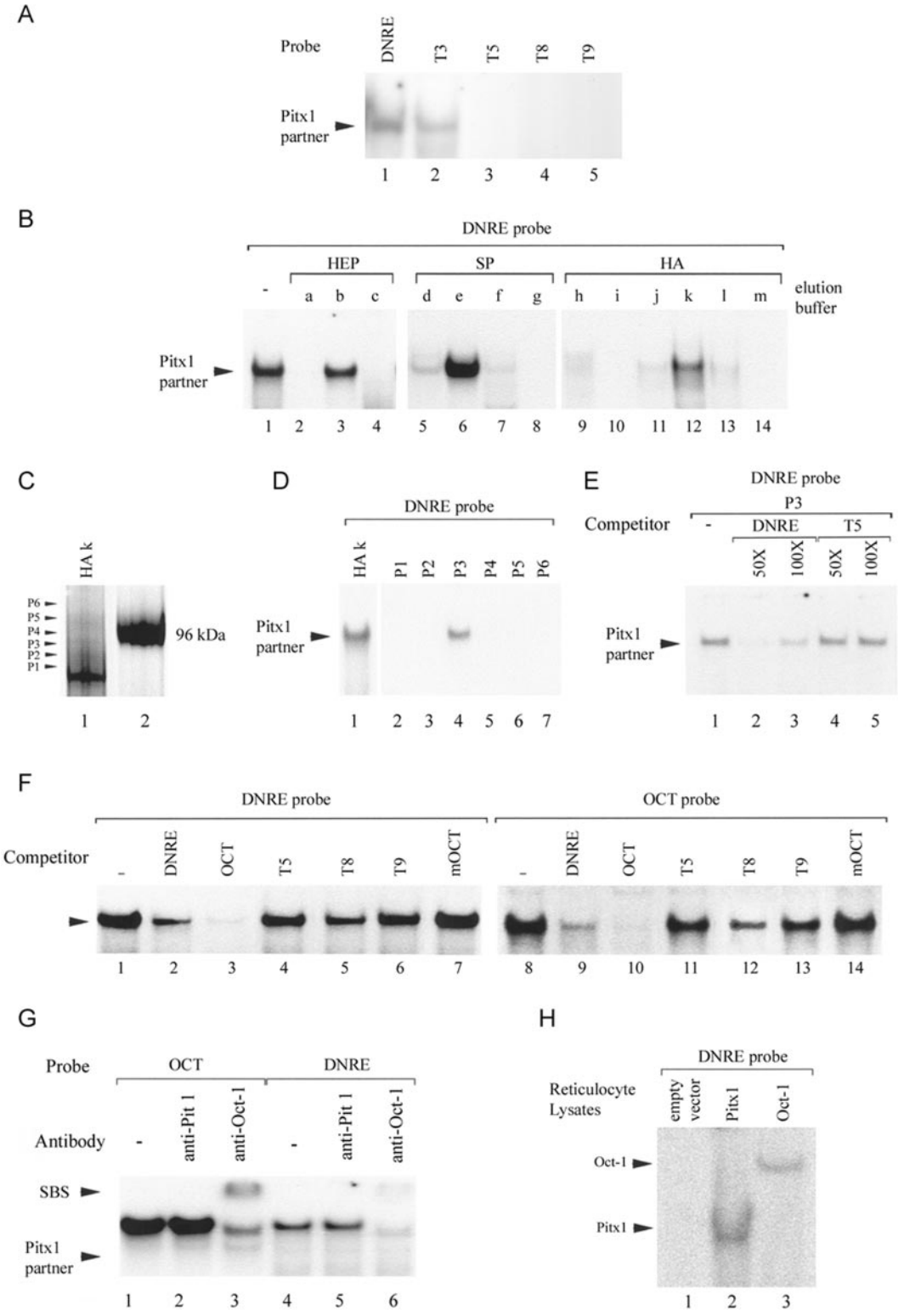

FIG. 2. Pitx1 partner binding activity to the DNRE leads to the identification of Oct-1. (A) Nuclear extracts were used for EMSA with five oligonucleotides: wild-type (26) and mutant T3, T5, T8, and T9 probes. Pitx1 partner binding is indicated by an arrowhead. (B) Chromatographic fractionation of Pitx1 partner binding activity to the DNRE. L929 nuclear extracts fractionated and eluted from chromatographic columns were used for EMSA with the DNRE probe. L929 nuclear extracts (lane 1, −) were fractionated successively with HEP, SP, and HA chromatographic columns. The fractionated extracts were eluted in the indicated buffers. The Pitx1 partner shifted band is indicated. (C and D) Positive fraction eluted in buffer k from chromatographic columns (HA k) was loaded on denaturing polyacrylamide gels containing 0.1% SDS (C, lane 1) in the presence of a molecular mass ladder (C, lane 2). After electrophoresis, the gel was silver stained and cut transversely into six fractions, designated

the HA column, which binds to the DNRE (Fig. 2B, lane 12), was loaded on a denaturing polyacrylamide gel containing 0.1% SDS (Fig. 2C, lane 1) and compared to a molecular mass ladder (Fig. 2C, lane 2). The gel containing the fractionated proteins was cut transversely, and proteins from each piece (P1 to P6) were eluted and renatured as described previously (35).

Binding activity of renatured proteins was assayed by EMSA using the DNRE probe (Fig. 2D). The P3 fraction (Fig. 2D, lane 4) binds to the DNRE, whereas P1, P2, P4, P5, and P6 (Fig. 2D, lanes 2, 3, 5, 6, and 7, respectively) do not bind to the DNRE. We concluded that the P3 fraction contains Pitx1 partner binding activity. The specific binding of the P3 fraction to the DNRE was demonstrated by efficient competition using the DNRE oligonucleotide (Fig. 2E, lanes 2 and 3), whereas no competition was observed using the T5 mutant oligonucleotide (Fig. 2E, lanes 4 and 5). The P3 fraction was therefore considered positive for the Pitx1 partner and could be a protein of a molecular mass close to 96 kDa, binding to the DNRE.

The sequence altered by mutation T5 contains the motif ATTTAAAG, which is almost identical to the octamer binding site (57). Thus, a POU family protein could bind the DNRE (Fig. 1C). Moreover, it has been demonstrated that Pitx1 can interact with Pit1 and act in synergy with this POU protein to regulate the pituitary expressed prolactin gene (48, 52). These data led us to identify the 92-kDa POU protein Oct-1 (46). We concluded that the Pitx1 partner could be related or identical to Oct-1.

To validate this hypothesis, we used EMSA-based experiments with the Oct-1 binding probe and antibodies directed against this protein. EMSA with the Oct-1 consensus binding site (OCT) in the presence of L929 nuclear extracts revealed that the OCT probe forms a protein-DNA complex with an electrophoretic mobility similar to that of the Pitx1 partner (data not shown). As the same mobility in a gel retardation assay does not necessarily mean that the same proteins are involved in complex formation, we investigated for specific binding (14). We realized competition assays using the DNRE (Fig. 2F, lanes 1 to 7) and OCT (Fig. 2F, lanes 8 to 14) probes, with an excess of the following competitors: DNRE, OCT, or the mutants T5, T8, T9, or mOCT. Pitx1 partner specific binding was demonstrated by efficient competition with the DNRE (Fig. 2F, lane 2), whereas no competition was observed with DNRE mutant oligonucleotides (Fig. 2F, lanes 4 to 6). Similarly, efficient competition was observed with the OCT oligonucleotide (Fig. 2F, lane 3) and not with the mOCT oligonucleotide (Fig. 2F, lane 7). Therefore, Pitx1 partner binding to the DNRE specifically competed with the Oct-1 consensus binding site. In reverse experiments, the specific nature of the retardation complex observed in EMSA by using the OCT probe with L929 nuclear extracts was demonstrated by efficient competition using the unlabeled wild-type OCT oligonucleotide (Fig. 2F, lane 10), whereas no competition was observed with the mOCT oligonucleotide (Fig. 2F, lane 14). Similarly, the DNRE (Fig. 2F, lane 9), but not T5, T8, or T9 (Fig. 2F, lanes 11 to 13), competes with OCT protein complex formation. These experiments suggest that DNRE and OCT probes specifically form a homologous retardation complex with L929 nuclear extracts in EMSA. As the OCT probe is described as specific for Oct-1 protein binding, we suggest that the Pitx1 partner could be related to the Oct-1 protein.

To confirm this hypothesis, we realized EMSA with OCT and DNRE probes in the presence of L929 nuclear extracts incubated with or without anti-Oct-1 antibody (Fig. 2G). These experiments revealed that the Pitx1 partner and the protein-DNA complex formed with the OCT probe are supershifted by anti-Oct-1 antibody (Fig. 2G, lanes 3 and 6) but not by anti-Pit1 antibody (Fig. 2G, lanes 2 and 5). We conclude that the Pitx1 partner is related or identical to the Oct-1 protein.

We therefore assessed recombinant in vitro-translated Oct-1 protein affinity for the DNRE (Fig. 2H). Oct-1 and Pitx1 proteins were produced using the TNT-coupled transcription-translation rabbit reticulocyte lysate system and incubated in presence of the DNRE ^{32}P-end-labeled probe. Pitx1 was used as a positive control for DNRE binding (Fig. 2H, lane 2). No binding was observed with empty vector (Fig. 2H, lane 1), whereas in vitro-transcribed and -translated Pitx1 and Oct-1 proteins shifted the DNRE probe (Fig. 2H, lanes 2 and 3, respectively). All together, these results indicate that the Oct-1 protein binds specifically to the DNRE of the IFN-A11 promoter in vitro.

Endogenous Oct-1 participates in repression of virus-induced IFN-A11 gene expression. In order to test the role of the endogenous Oct-1 protein in the repression of IFN-A11 promoter activity, NDV-induced L929 cells were transfected with the Oct-1 antisense RNA expression vector in the presence of the IFN-A11/luciferase reporter construct (Fig. 3A). Luciferase activity was evaluated as described in Materials and Methods. Overexpression of Oct-1 antisense RNA led to a significant increase in IFN-A11 promoter activity after virus induction (Fig. 3A). In order to determine whether this effect was due to specific knockdown of the Oct-1 protein, the Oct-1 sense expression vector was cotransfected with the Oct-1 antisense RNA expression vector in the presence of the IFN-A11/luciferase reporter construct. Here we show that the derepression due to antisense RNA expression was partially abolished in a dose-dependent manner when Oct-1 sense was expressed in NDV-induced L929 cells (Fig. 3A). In contrast, Oct-1 antisense RNA led to no significant change in IFN-B promoter

P1 to P6 (indicated with arrowheads). After renaturation, DNA binding proteins separated by PAGE were used in EMSA with the DNRE probe (D) (see also Materials and Methods). HA k was used as a control (D, lane 1). Pitx1 partner binding is indicated. (E) Specific binding of the P3 fraction to the wild-type DNRE probe. The P3 fraction, as described for panel D, was incubated with the DNRE probe and a 50-fold (50×) or 100-fold (100×) molar excess of unlabeled DNRE or T5 oligonucleotides. No competitor was used for lane 1. Pitx1 partner binding is indicated. (F) Specific binding of the Pitx1 partner to DNRE and OCT probes. By EMSA, L929 nuclear extracts were incubated with DNRE and Oct binding consensus probe OCT(−) and a 100-fold molar excess of unlabeled DNRE, OCT, T5, T8, T9, or mOCT oligonucleotides. (G) The Pitx1 partner is related or identical to the Oct-1 protein. Nuclear extracts from L929 cells were incubated with OCT and DNRE probes (−) and anti-Pit1 and anti-Oct-1 antibodies. Pitx1 partner shifted bands and supershifted bands (SBS) are indicated by arrowheads. (H) In vitro-transcribed and -translated recombinant Oct-1 binds to the DNRE. Reticulocyte lysates containing the in vitro-transcribed and -translated proteins Pitx1 and Oct-1 were used for EMSA in the presence of the DNRE probe. Empty vector was used as the negative control. Oct-1 and Pitx1 shifted bands are indicated.

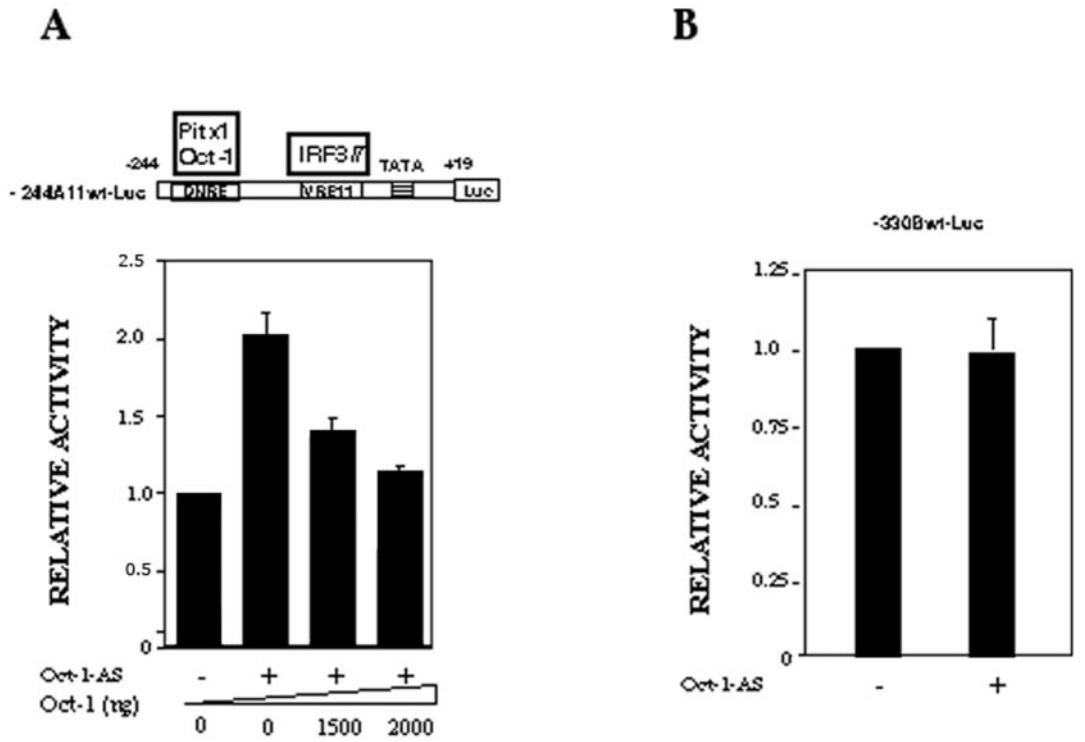

FIG. 3. Endogenous Oct-1 participates in repression of virus-induced IFN-A11 gene expression. (A) Overexpression of Oct-1 antisense RNA led to a significant increase in IFN-A11 promoter activity. The −244A11wt promoter was transfected in NDV-induced L929 cells with the indicated expression vectors (see also Fig. 1B). The map of −244A11wt is shown at the top. One microgram of Oct-1 antisense RNA expression vector (Oct-1-AS) was added when indicated. Oct-1 cDNA expression vector (Oct-1) was added (1.5 or 2 μg) in the presence of Oct-1-AS in induced cells. Transcription activity after virus induction is relative to that of IFN-A11, which was set at 1. (B) Overexpression of Oct-1 antisense RNA led to no significant change in IFN-B promoter activity. The −330 IFN-B promoter was transfected in NDV-induced L929 cells with the indicated expression vector. One microgram of Oct-1-AS was added when indicated. Transcription activity after virus induction is relative to that of IFN-B, which was set at 1.

activity (Fig. 3B). This indicates that endogenous Oct-1 plays a role in NDV-induced IFN-A11 repression.

Oct-1 represses IRF-3 and IRF-7 transcription activation of the IFN-A11 gene. Since IRF-3 and IRF-7 are the main activators of IFN-A genes, we determined whether the repressive effect of Oct-1 on the IFN-A11 promoter could be mediated by repression of the transcriptional activities of IRF-3 and IRF-7. The effect of Oct-1 on the activation of the IFN-A11 promoter by overexpression of IRF-3 or IRF-7 was tested with a combination of Oct-1 and either the IRF-3 or the IRF-7 expression vector in the context of the wild-type IFN-A11 or T5 mutant promoter in L929 cells with or without NDV induction (Fig. 4). IRF-3 and IRF-7 were able to activate both wild-type and mutant T5 promoter transcription. Moreover, IRF-7 overexpression activated mock-induced activity, compared to basal activity. No significant difference between mock-induced activities was observed with or without IRF-3 overexpression. Oct-1 overexpression repressed the activation by IRF-3 and IRF-7 in the context of the wild-type promoter, both in mock-induced and NDV-induced conditions. In contrast, Oct-1 overexpression had no effect on IRF-3 or IRF-7 activation of the T5 mutant promoter. Together, these results suggest that Oct-1 inhibits IRF-3 and IRF-7 activation of the IFN-A11 promoter and that its repressive activity strictly depends on its binding to the promoter.

The POU homeodomain protein Oct-1 represses IFN-A11 transcription with Pitx1. As Oct-1 was shown to repress IRF-3 and IRF-7 activation of IFN-A11 expression and since we showed that the Pitx1 homeodomain protein represses the IFN-A11 promoter (20, 25), we tested whether these two factors could act together for repression of activator factors. The importance of Oct-1 and Pitx1 in IFN-A11 promoter activity was tested by transient transfection in NDV-induced L929 cells with a combination of Pitx1 and Oct-1 expression vectors in the presence of IRF-3 and IRF-7 (Fig. 5). It is possible that Oct-1 and Pitx1 homeodomain proteins, binding to a contiguous site within the DNRE, may act together in order to repress IFN-A11 gene expression. As previously described, IRF-3 and IRF-7 overexpression activated IFN-A11 promoter activity (Fig. 5A). Moreover, IRF-7 was able to activate the promoter in the absence of virus induction. In mock-induced conditions, in the presence of IRF-3 overexpression, Pitx1 and Oct-1 significantly repressed the IFN-A11 promoter, whereas individual expression did not modify the promoter activity. In contrast, Oct-1 represses IFN-A11 promoter activation by IRF-3 under NDV-induced conditions. As previously described, Pitx1 by itself is also able to repress IFN-A11 promoter activation by IRF-3 under these conditions (20). Interestingly, overexpression of Pitx1 and Oct-1 led to an additive repression of the IFN-A11 promoter activated by IRF-3 and IRF-7 in induced

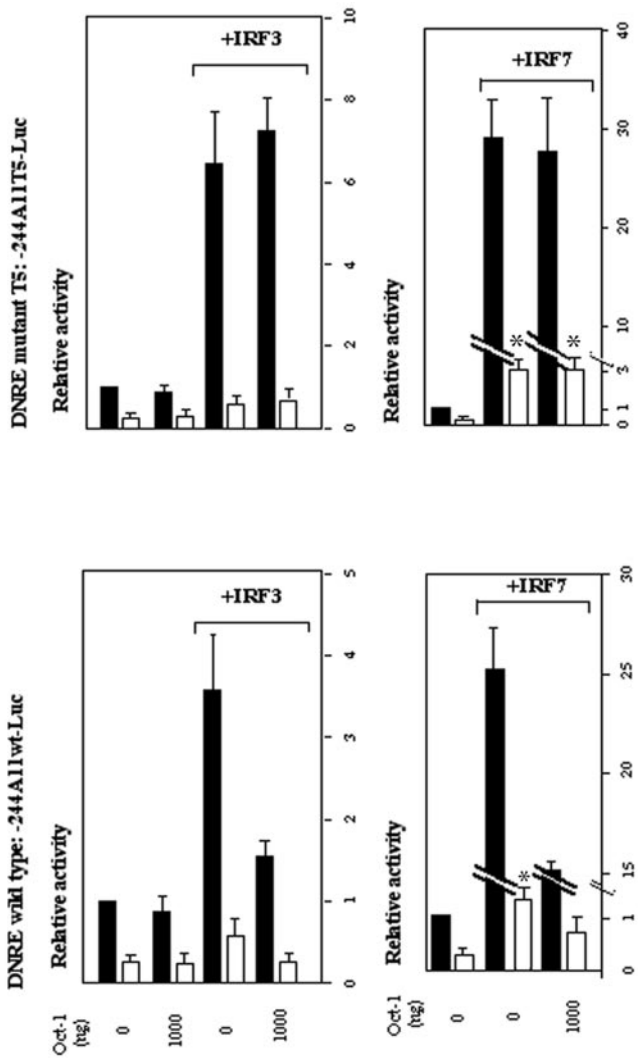

FIG. 4. Oct-1 represses IRF-3 and IRF-7 transcriptional activation of the IFN-A11 gene. Cells were transfected with the −244A11wt or T5 mutant (−244A11T5) reporter plasmid and either Oct-1, IRF-3, or IRF-7 expression vector (1 μg each) and were mock induced (open bars) or NDV induced (filled bars) as described in Materials and Methods. Oct-1 represses wild-type IFN-A11 but not T5 mutant transcriptional activities when IRF-3 or IRF-7 is overexpressed. Transcriptional activities are expressed relative to that of each promoter. Mock-induced activities significantly different from basal activity are indicated with asterisks.

cells. In mock-induced conditions, Pitx1 and Oct-1 expression was able to repress IRF-7 activation of the IFN-A11 promoter to levels similar to basal activity levels. Similarly, individual expression of Pitx1 or Oct-1 significantly repressed IRF-7 activation of the promoter induced by the virus. Moreover, overexpression of both of them led to an additive repression of IFN-A11 promoter activity. These results suggest that Pitx1 and Oct-1 repressive activities upon the IFN-A11 induced promoter are additive. In order to detect functional interactions between Pitx1 and Oct-1, IFN-A11 promoter activity was tested by transient transfection in NDV-induced L929 cells with a combination of Pitx1 and Oct-1 expression vectors in the presence of IRF-7 (Fig. 5B). Here we show that Oct-1 repressed IRF-7 activation of the IFN-A11 promoter in a dose-dependent manner. Moreover, in the presence of limiting amounts of the Pitx1 expression vector, Oct-1 and Pitx1 repressive activities were additive.

All together, these results suggest that Oct-1 is able to repress IRF-mediated activation of the IFN-A11 promoter and that Oct-1 and Pitx1 additively repress the promoter activated by IRF overexpression. Given that Oct-1 and Pitx1 bind to neighboring sites within the DNRE and that they show additive repression of the IRF-activated IFN-A11 promoter-mediated expression, we investigated the binding relation between these two transcription factors.

Oct-1 and Pitx1 do not physically interact and they do not show cooperation of binding to the DNRE. First, we investigated whether Oct-1 and Pitx1 are able to physically interact. Direct interaction was tested by pulldown assays performed using in vitro-transcribed and -translated ^{35}S-labeled Oct-1, positive control IRF-3 or IRF-7, or Luc protein, as well as MBP fusion protein (MBP-Pitx1 or MBP-LacZ as the control), as described previously (20). ^{35}S-labeled Oct-1 and luciferase do not interact with MBP-Pitx1 (Fig. 6A, lanes 8 to 14), in contrast to ^{35}S-labeled IRF-3 and IRF-7 (Fig. 6A, lanes 1 to 7). These data suggest that in these conditions Oct-1 and Pitx1 do not physically interact.

As pulldown assays are not efficient to measure indirect interactions, we tested whether endogenous Oct-1 is able to interact with Pitx1 in L929 cells by coimmunoprecipitation assays. Nuclear extracts were prepared and precipitated with antibody directed against the Oct-1 protein as described elsewhere (20). Immunoprecipitate was tested for Pitx1 presence by Western blotting. Recently we have shown that, under these conditions, antibodies directed against IRF-3 and IRF-7 coimmunoprecipitate the Pitx1 protein (20). In contrast, no signal was detected with anti-Oct-1 antibody (Fig. 6B, lane 4). All together, these results suggest that we are not able to stress any interaction, direct or indirect, between Oct-1 and Pitx1.

As Oct-1 and Sp1 were demonstrated as cooperatively binding to U2 RNA gene promoters (45), we tested whether Oct-1 could facilitate Pitx1 DNA binding. In vitro-transcribed and -translated Oct-1 and Pitx1 proteins were used for EMSA with the ^{32}P-labeled DNRE probe separately or together. Pitx1 and Oct-1 were able to bind the DNRE separately (Fig. 6C, lanes 1 and 2), and when Oct-1 and Pitx1 were incubated together with the DNRE and submitted to gel migration, no difference was observed in Oct-1 or Pitx1 binding efficiencies (Fig. 6C, lane 3). Similarly, Oct-1 binding to the DNRE in nuclear extracts was not altered by anti-Pitx1 antibody incubation (data not shown). We conclude that Oct-1 and Pitx1 do not physically interact and that they do not show cooperation of binding to the DNRE.

Oct-1 binds in vivo to the endogenous IFN-A11 promoter in mock-induced and NDV-induced L929 cells. Since our results indicate that Oct-1 and Pitx1 bind to the DNRE in vitro, we sought to determine whether these factors bind to the endogenous IFN-A11 promoter in mock-induced and NDV-induced L929 cells by use of chromatin immunoprecipitation assays (Fig. 7). L929 cells were mock induced or induced with NDV, and 8 h postinduction, proteins were cross-linked to DNA and the protein-DNA complexes were precipitated with specific antibody in two separate experiments (Fig. 7A). The DNA

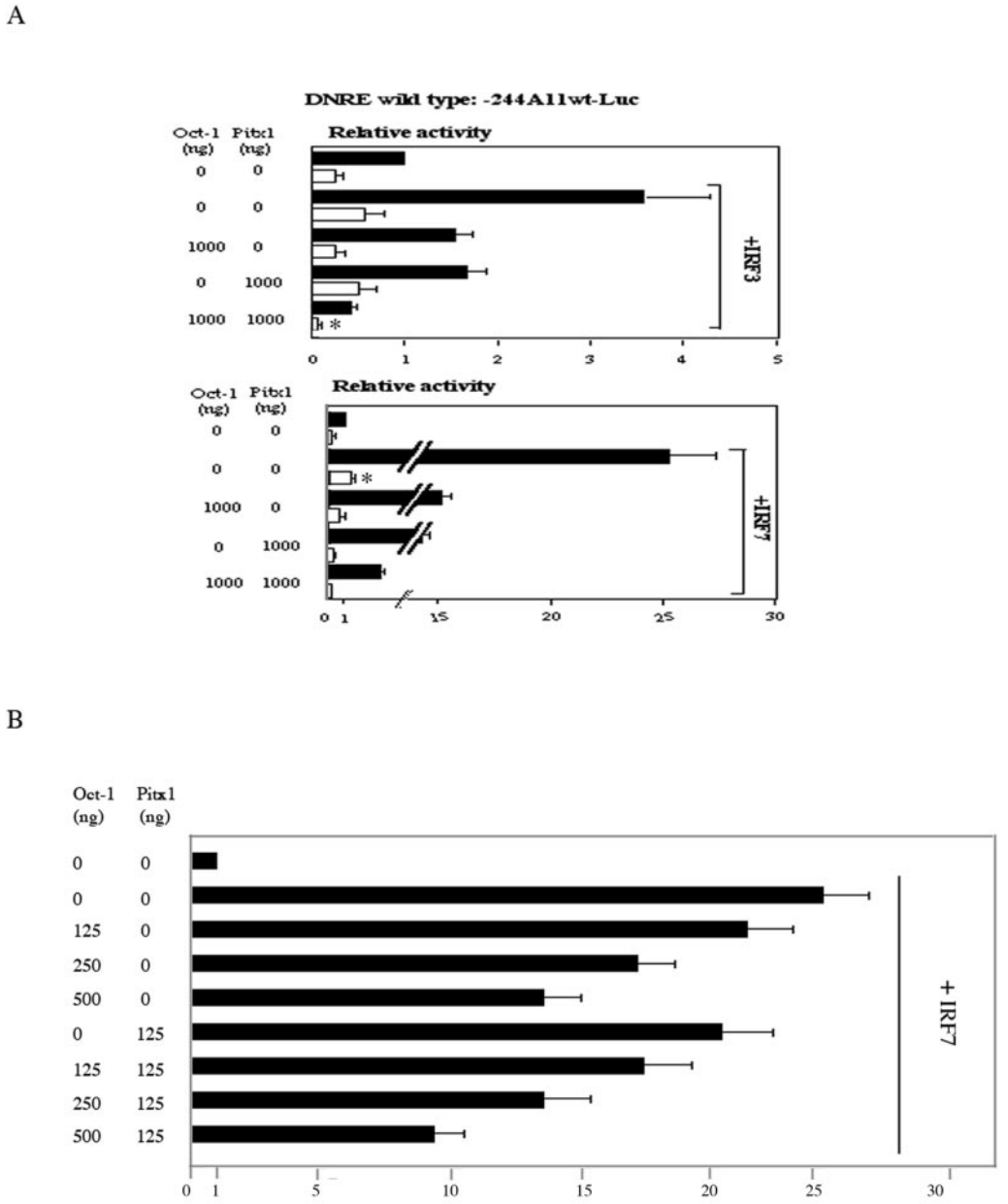

FIG. 5. Oct-1 and Pitx1 cooperate to repress IRF-3 and IRF-7 activation of IFN-A11 expression. (A) L929 cells were transfected with the −244A11wt promoter and a combination of Pitx1 or Oct-1 and IRF-3 or IRF-7 expression vectors (1 μg each) and were mock induced (open bars) or NDV induced (filled bars). Oct-1 and Pitx1 repressive effects are additive upon IFN-A11 activation by IRF-3 or IRF-7 overexpression. Mock-induced activities significantly different from basal activity are indicated with asterisks. (B) NDV-induced L929 cells were transfected with a combination of 1 μg IRF-7 and various amounts of Oct-1 or Pitx1 expression vector. Transcription activity after virus induction is relative to that of IFN-A11, which was set at 1.

present in the precipitates was then purified and radioactively amplified by PCR with specific primers recognizing the IFN-A11 subtype promoter (−457 to −113) or with primers specific for pericentromeric gamma-satellite DNA amplification. In mock-induced L929 cells, the endogenous promoter was amplified from the DNA immunoprecipitated with antibody directed against Oct-1, whereas no amplification was observed with anti-Pitx1, anti-IRF-3, and anti-IRF-7 antibodies. In NDV-induced L929 cells, amplification was observed with anti-Oct-1, anti-Pitx1, anti-IRF-3, and anti-IRF-7 antibodies, indicating that these factors bind to the IFN-A11 promoter region in vivo in L929 cells 8 h after NDV induction. Under both conditions, irrelevant antibody led to no amplification (data not shown). Moreover, specific IFN-A11 promoter amplification was tested by nonradioactive PCR amplification and sequencing (data not shown). Since Oct-1 binding to the IFN-A11 promoter was detected under both conditions (Fig. 7B, lanes 1 to 4), we investigated for specific immunoprecipitation with anti-Oct-1 antibody. Indeed, by use of primers designed to amplify pericentromeric gamma-satellite DNA, no amplifica-

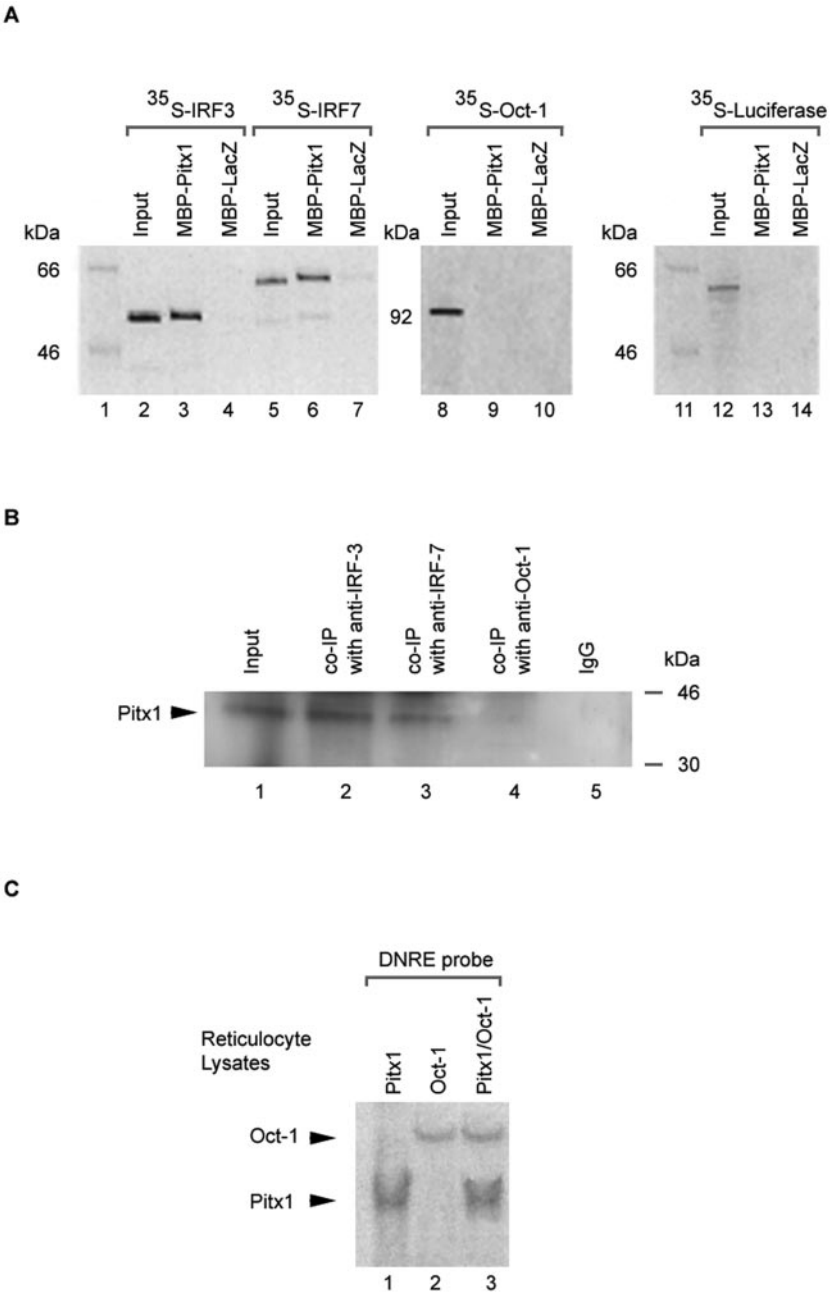

FIG. 6. Oct-1 and Pitx1 do not physically interact and they do not show cooperation of binding to the DNRE. (A) Direct interactions were tested by pulldown assays performed using MBP fusion proteins (MBP-Pitx1 and MBP-LacZ) and in vitro-translated ^{35}S-labeled IRF3, IRF7, Oct-1, and luciferase proteins. An aliquot of input protein corresponding to 10% of labeled protein used in the assay is shown for comparison. Molecular mass markers are indicated in lanes 1 and 11. (B) Nuclear extracts from L929 cells were used for coimmunoprecipitation (co-IP) with the indicated antibodies. Input and control samples are presented. Western blot analysis of the proteins shows that Pitx1 was specifically bound to IRF-3 and IRF-7. Molecular mass markers are indicated at the right. (C) In vitro-transcribed and -translated recombinant Oct-1 and Pitx1 bind to the DNRE. Reticulocyte lysates containing the in vitro-transcribed and -translated proteins Pitx1 and Oct-1 were used for EMSA with the DNRE probe separately (lanes 1 and 2) or together (lane 3). Oct-1 and Pitx1 shifted bands are indicated.

tion was observed from DNA immunoprecipitated with anti-Oct-1 antibody under either condition (Fig. 7B, lanes 5 to 8), whereas the control was positive for amplification (lane 9) (described in reference 44). All together, these results indicate that (i) Oct-1 binds to the IFN-A11 promoter in vivo under mock-induced conditions and 8 h postinduction and (ii) Pitx1, IRF-3, and IRF-7 bind the promoter after induction.

Oct-1-deficient MEFs exhibit increased endogenous IFN-A gene expression and modification in the pattern of IFN-A subtypes. The importance of endogenous Oct-1 in IFN-A pro-

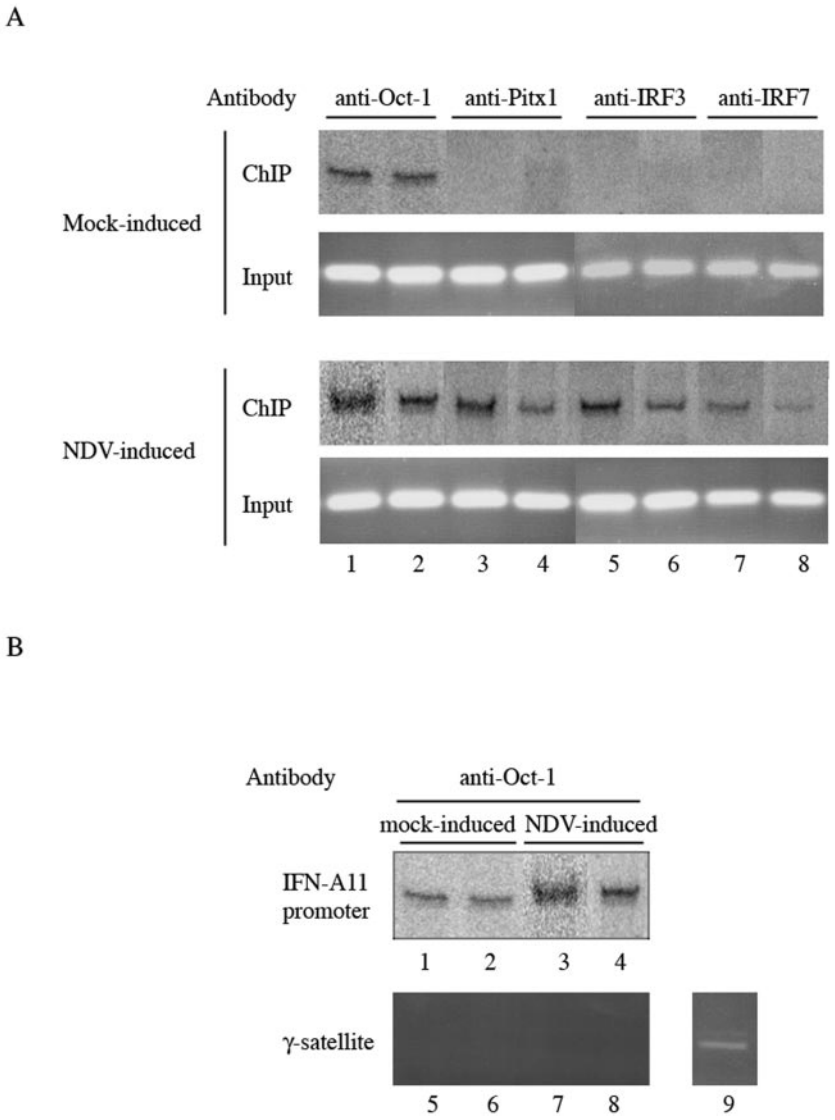

FIG. 7. Oct-1 binds in vivo to the endogenous nuclear IFN-A11 promoter. (A) In vivo levels of Oct-1, Pitx1, IRF-3, and IRF-7 binding to the endogenous IFN-A11 promoter were analyzed by protein-DNA immunoprecipitation with mock-induced and NDV-induced L929 cells. Immunoprecipitations were performed with the indicated antibodies. DNA recovered from protein-DNA immunoprecipitation was amplified using primers specific for IFN-A11. Results from input DNA and chromatin immunoprecipitation assays (ChIP) are presented. Two separate experiments are presented. (B) DNA recovered from Oct-1 DNA immunoprecipitation was amplified using primers specific for IFN-A11 or specific for pericentromeric gamma-satellite DNA (γ-satellite). A positive control for γ-satellite amplification is presented (lane 9) (44).

moter activity was tested by using Oct-1-deficient MEFs. As previously described (55), Oct-1-deficient MEFs led to a significant decrease in Oct-1 DNA binding activity by using the OCT probe (data not shown).

To characterize the role of Oct-1 in IFN-A gene repression after virus induction, we quantitatively and qualitatively compared the endogenous IFN-A expression levels of wild-type MEFs and Oct-1-deficient MEFs. Total RNAs from both cell types were isolated before and 8 h after virus induction for quantification by RT-PCR using IFN-A-specific primers. The levels of IFN-A mRNA were quantified using serial dilution RT-PCR (Fig. 8A). mRNA expression from wild-type MEFs was poorly detected following 1/5 dilution and hardly detected following 1/25 dilution (Fig. 8A, lanes 2 and 3). In contrast, IFN-A mRNA from Oct-1-deficient MEFs was clearly detected following 1/25 dilution and was still visible following 1/125 dilution (Fig. 8A, lanes 7 and 8). Differences were significant as shown by GAPDH amplification. In both cell lines, IFN-A mRNA were undetectable in the absence of virus induction (data not shown). Thus, induction of IFN-A gene expression was significantly increased after virus induction in Oct-1-deficient MEF cells.

To distinguish IFN-A expressed in wild-type and Oct-1-deficient MEFs, cDNAs were cloned. Clones were randomly selected and sequenced for each cell line (Fig. 8B). As expected, IFN-A gene expression displayed a mixture of distinct subtypes. In wild-type MEFs, IFN-A4 and IFN-A2 were the two most abundant species detected. Strikingly, IFN-A4 was the

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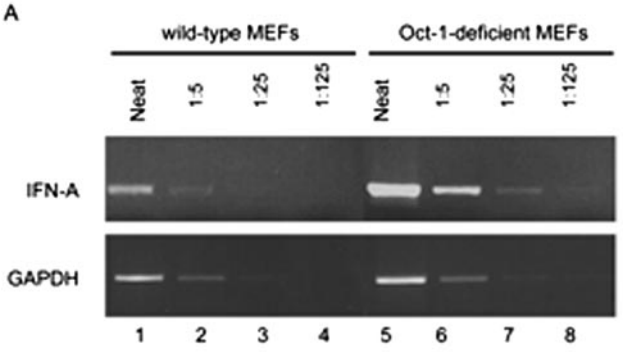


B

Representation of IFN-A subtypes in NDV induced wild-type and Oct-1 deficient immortalized MEFs

	N° of clones with subtype	
IFN-A subtype	wt MEFs	Oct-1 -/- MEFs
A4	12	39
A2	12	1
A5	1	4
A14	1	-
Total no. of clones sequenced	26	44

FIG. 8. Oct-1-deficient MEFs exhibit increased endogenous IFN-A gene expression and modification in the pattern of IFN-A subtypes. (A) Absence of Oct-1 led to an increase in endogenous IFN-A expression. Expression and quantification of IFN-A genes 8 h after virus induction were monitored using serial dilution RT-PCR with conserved consensus primers for IFN-A in wild-type and Oct-1-deficient MEFs. GAPDH expression was used as a control. Undiluted samples are indicated (Neat). (B) Representation of IFN-A subtypes expressed in wild-type and Oct-1-deficient MEFs 8 h after virus induction. RT-PCR products were cloned, and clones were randomly sequenced.

most abundant species detected for Oct-1-deficient MEFs. These results suggest that endogenous Oct-1 not only quantitatively affects IFN-A gene expression but also differentially regulates IFN-A subtypes, its absence resulting in altered patterns of IFN-A gene expression.

Effective and potent octamer-like sequences are broadly represented within IFN-A promoters. As Oct-1-deficient MEFs exhibit increased IFN-A expression, we investigated for the presence of octamer-like sequences within other IFN-A promoters. Indeed, EMSAs with OCT, DNRE, and oligonucleotides from IFN-A7 and IFN-A5 promoters in the presence of L929 nuclear extracts revealed protein-DNA complex formation with similar levels of electrophoretic migration (Fig. 9A). In order to confirm that complexes contain Oct-1, we performed EMSA in the presence of nuclear extracts incubated with anti-Pit1 or anti-Oct-1 antibodies. Complexes are supershifted by anti-Oct-1 antibody (Fig. 9A, lanes 3, 6, 9, and 12) but not by anti-Pit1 antibody (Fig. 9A, lanes 2, 5, 8, and 11). We conclude that Oct-1 binds to IFN-A5 and IFN-A7 promoters in vitro. Furthermore, sequence analyses revealed that an Oct-1 binding site similar to that for IFN-A11 and IFN-A7 is conserved within IFN-A4, IFN-A2, IFN-A9, and IFN-A14 promoters (Fig. 9B). Moreover, by homology with the IFN-A5 promoter, we identified potent Oct-1 binding sites within the IFN-A1 promoter. These results suggest that of approximately 15 IFN-A genes, 8 contain octamer-like sequences within their promoters. Together with the analysis of the IFN-A subtypes expressed in Oct-1-deficient MEFs, these results suggest that Oct-1 could be implicated in transcription regulation of multiple IFN-A genes.

Oct-1-deficient MEFs exhibit increased antiviral activity. Since Oct-1-deficient MEFs exhibit increased endogenous IFN-A gene expression compared to wild-type MEFs, we investigated if this difference is sufficient to promote cytological effects. We compared the capacities of VSV infection to induce cell death in L929 cells previously treated with NDV-induced supernatant from wild-type and Oct-1-deficient MEFs. The supernatants were collected 8 h after infection and added, after serial twofold dilution, to L929 cells. VSV was added 24 h after supernatants, and cell survival was measured by vital dye staining. As shown in Fig. 10, cell survival was enhanced by incubation with supernatant from Oct-1-deficient MEFs, compared to cell survival for wild-type MEFs. These results indicate that cell resistance to the VSV cytopathic effects conferred by the supernatants is dependent on the Oct-1 state in MEFs. These results suggest that Oct-1 is able to regulate antiviral activity in MEFs.

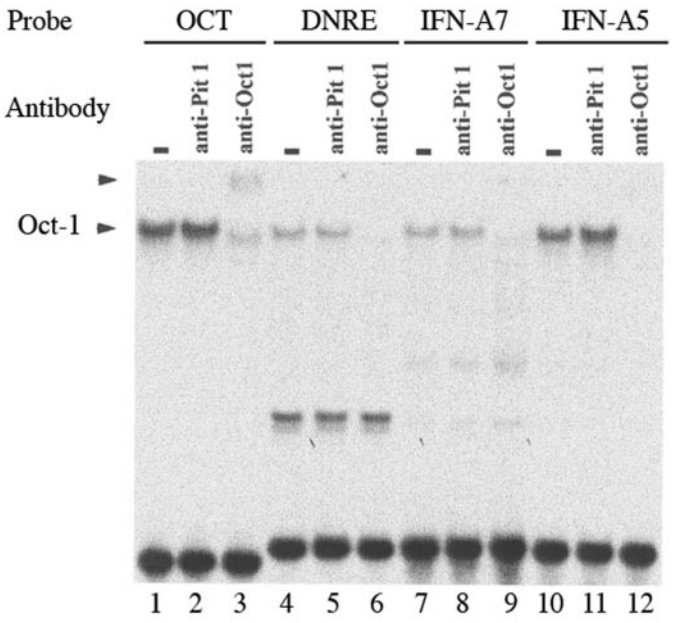
DISCUSSION

The analysis of virus-induced IFN-A gene expression led us to study the POU transcription factor Oct-1. For the first time, we show here that Oct-1 plays a role in the differential repression of these genes.

The POU transcription factor Oct-1 is ubiquitously expressed (46) and is involved in the transcription regulation of numerous genes, among which are H2B (10), small nuclear RNAs (11, 50), and Ig heavy chain and kappa light chain genes (12, 36). Oct-1 can also repress von Willebrand factor (42), VCAM-1 (17, 18), or interleukin-8 expression (57, 58). Oct-1 can therefore act as a repressor or an activator of transcription. On the other hand, the Oct-1 protein is essential for the initiation of adenovirus DNA replication (31, 37, 39, 40). Recently, it has been shown that Oct-1 deficiency is involved in embryonic lethality, decreased erythropoiesis, and defective octamer-dependent promoter activation in mice (55). Furthermore, herpes simplex virus infections are arrested in Oct-1-deficient cells (32). From our study, it would be of interest to determine whether IFN-A expression is implicated in this antiviral effect. Finally, the Oct-1 protein is dispensable for B-cell development and Ig transcription (56).

We recently described Pitx1 as a repressor of NDV-induced IFN-A gene expression (20, 25). Here we show that the purification of a DNRE binding protein led to the identification of a homeoprotein member of the POU family, Oct-1. Mutations within the DNRE that inhibit the repressive effect of the wild-type DNRE abolish Oct-1 binding in vitro. Moreover, Oct-1 antisense RNA expression specifically increases IFN-A11 promoter activity. In contrast, Oct-1 antisense RNA expression

A



B

IFN-A11	ACCATTTAAGTCTA	ATTTAAAG	TGAA	Effective Oct-1 binding sites
IFN-A7	ACACATATAGTGAA	ATTTAATG	AAAA	
IFN-A4	ACAATTTAAGTGTA	ATTTAAAG	AGAA	Potent Oct-1 binding sites
IFN-A2	ACAATTTAAGTTTA	ATTTAAAG	TGAA	
IFN-A9	TAAATATTGAGAAA	ATTTAAAG	TATA	
IFN-A14	ACTTTACTGAAAAA	ATTTAAAG	TATA	
IFN-A5	ATGAAA	ATTCAAAT	TAGGAAGAAAA	Effective Oct-1 binding site
IFN-A1	ATGAAA	ATTCAAAT	TAGGAAAAGAA	Potent Oct-1 binding site

FIG. 9. Effective and potent octamer-like sequences are broadly represented within IFN-A promoters. (A) Nuclear extracts were used for EMSAs with OCT, DNRE, and IFN-A7 and IFN-A5 promoter oligonucleotides and incubated with anti-Pit1 or anti-Oct-1 antibody. Oct-1 binding is indicated by arrowheads. Supershifted complexes are shown. (B) Sequence alignment showing octamer-like sequence conservation between effective and potent Oct-1 binding sites. Conserved Oct-1 binding sites are framed. The nucleotide substitution in the octamer-like sequence is underlined.

has no effect on IFN-B induction. These results are consistent with the analysis of the effects of mutations in the octamer motif within the human IFN-B promoter (9). Indeed, several mutations that decreased Oct-1 binding had no influence upon the level of virus induction. Moreover, sequence comparison between murine and human IFN-B promoters indicates that the Oct-1 binding site is not conserved in the murine promoter. On the other hand, Oct-1 proteins from mice and humans should not be directly compared (47). Oct-1 is expressed in vivo as several isoforms, resulting from alternative splicing (6,

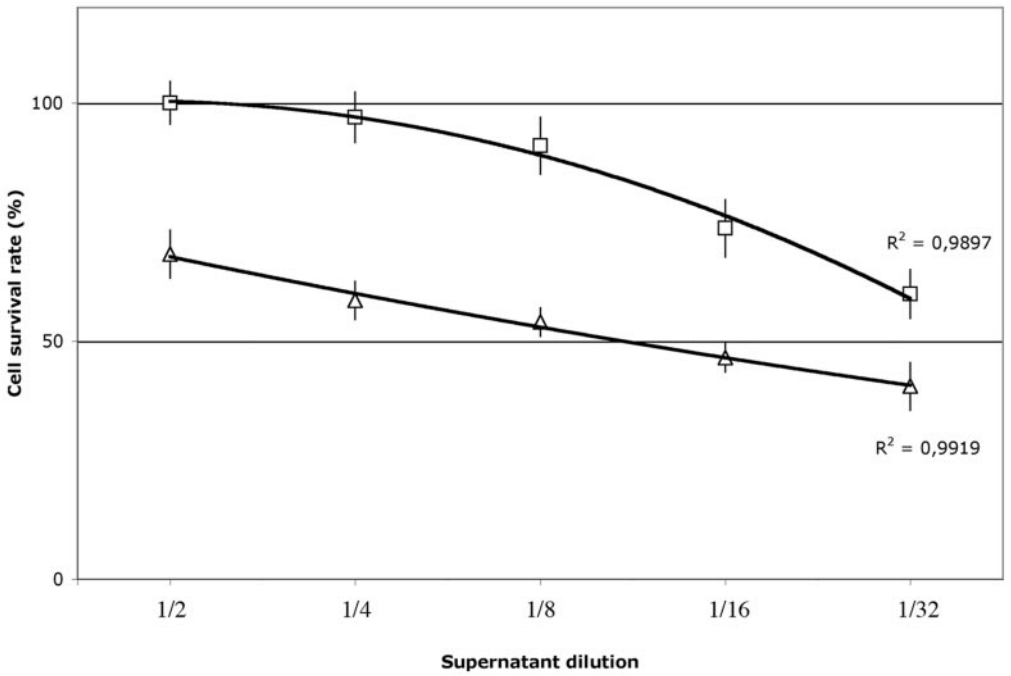


FIG. 10. Oct-1-deficient MEFs exhibit increased antiviral activity. L929 cells were incubated with supernatants from wild-type (triangles) and Oct-1-deficient MEFs (squares) and challenged with VSV. NDV-induced supernatants were collected 8 h after infection and added, after serial twofold dilution, to L929 cells. VSV was added 24 h after supernatants, and cell survival was measured by vital dye staining. Cell survival rate is relative to that of L929 in the absence of VSV, set at 100%. Means and standard errors determined from at least four experiments are shown. Projection curves are shown with their coefficients of determination (R^2).

21, 59). It would be of interest to determine whether these isoforms are equally implicated in IFN-A gene repression after virus induction. Furthermore, Oct-1 physically interacts with several proteins (24, 27, 54), and different regions of the protein are implicated in these interactions (7). The transcription-repressive effects of Oct-1 on IFN-A gene expression after virus induction may be due to a specific region of the protein. We will investigate for specific regions implicated in Oct-1 repressive effects on IFN-A gene transcription.

Notably, Oct-1 is the second homeodomain protein which binds the DNRE and represses NDV-induced IFN-A11 gene expression activated by IRF-3 or IRF-7. Indeed, we demonstrated that Pitx1 binds the DNRE in the murine IFN-A11 promoter and represses expression of this gene. Here we show that Oct-1 and Pitx1 bind in vivo to the endogenous IFN-A11 promoter (Fig. 7). However, we do not show any physical interaction between Oct-1 and Pitx1, either by pulldown assays or by coimmunoprecipitation (Fig. 6). Furthermore, we could not demonstrate any competition or facilitation for DNRE binding between these two factors, whereas they bind neighboring sites within the DNRE. Finally, in transfection assays, Oct-1 and Pitx1 repressive effects are additive.

Whereas Pitx1 repression of IFN-A gene expression was likely due to Pitx1 physical interaction with IRF-3 and IRF-7 (20), the Oct-1 repression mechanism remains unknown. We now show that the Oct-1 protein can repress IRF-3 and IRF-7 activation of NDV-induced IFN-A expression. Moreover, Oct-1 contains a POU DNA binding domain, which is composed of a POU-specific domain and a POU homeodomain (4, 8, 15, 46). As for Pitx1, the Oct-1 homeodomain could mediate interaction with IRF-3 and IRF-7. In contrast, Oct-1 can prevent transcription activation by binding to an OCT element that overlaps the CCAAT/enhancer binding protein activator binding site within the interleukin-8 promoter (57). On the other hand, Oct-1 was described as a histone deacetylase (HDAC) target which maintains HLA-DRA promoter repression in Rb-defective cells and prevents NF-Y activator binding to the promoter (33, 34). We previously discussed that trichostatin A, an inhibitor of HDACs, activates IFN-A expression and that the Pitx1 repressive effect is not related to HDAC activity (20). We concluded that an unknown factor must recruit HDAC to the IFN-A promoter. Therefore, we will investigate whether Oct-1 represses IFN-A gene expression through chromatin modifications or through direct interference with activators IRF-3 and IRF-7.

Oct-1 is phosphorylated by different kinases in vitro (13, 19) or in a cell cycle-dependent manner (38, 43). On the other hand, Oct-1 phosphorylation is induced by stress stimuli and DNA damage (41). Interestingly, the Oct-1 protein can be phosphorylated by DNA-dependent protein kinase (41, 53), which also phosphorylates IRF-3 (23). Notably, its phosphorylation can regulate its DNA binding activity (13, 43). Furthermore, Oct-1 is postranslationally modified and its DNA binding activity is regulated during herpes simplex virus 1 infection (1). In contrast, in vivo Oct-1 binding to the endogenous IFN-A11 promoter seems not to be regulated during NDV infection, because Oct-1 binding to the IFN-A11 promoter was detected in mock-induced and virus-induced L929 cells. We will investigate Oct-1 regulation in NDV-induced cells and

how this regulation may influence the Oct-1 repressive effect on IFN-A gene expression.

The ability of Oct-1 to regulate expression of proteins involved in cell cycle regulation (3, 10, 27), apoptosis (16, 22), and immunity (5, 12, 17, 18, 33, 34, 36, 58) and Oct-1 activation in response to stress signals (16, 22, 41, 60), including viral infection (1), suggest an important role for Oct-1 in a defense mechanism against cellular stress. Accordingly, here we show that Oct-1-deficient MEFs exhibit increased endogenous IFN-A gene expression and modification in the pattern of IFN-A subtypes after virus induction. Among IFN-A subtypes expressed in Oct-1-deficient MEFs after virus infection, IFN-A11 is not represented. This result is not surprising for several reasons. First, IFN-A promoter strength is a combination of activator and repressor sequences, and we demonstrated previously that activator sequences from IFN-A11 are mutated compared to those from IFN-A4 (2). Second, we demonstrated that effective and potent octamer-like sequences are broadly represented within IFN-A promoters. Indeed, we show that Oct-1 binds in vitro to IFN-A5 and IFN-A7 and suggest potent Oct-1 binding sites in IFN-A1, IFN-A2, IFN-A4, IFN-A9, and IFN-A14. Finally, we previously demonstrated that Pitx1 is implicated in IFN-A11 repression, and our results suggest that Pitx1 and Oct-1 repression is additive upon IFN-A11 expression. Therefore, the absence of detectable IFN-A11 expression in Oct-1-deficient MEFs could be due to Pitx1 repression or to the weakness of VRE-A11.

From our study, we conclude that (i) Oct-1 is implicated in the regulation of IFN-A gene transcription and (ii) Oct-1 deficiency is sufficient to increase cytological antiviral activity in MEFs. The broad representation of functional and potent octamer-like sequences within IFN-A promoters suggests that Oct-1 could be a general IFN-A regulatory transcription factor. IFN-α has been widely used in the treatment of solid and hematological malignancies. IFN-α-based combinatorial therapies associate IFN-α and therapeutic agents (30, 49). It was recently shown that Oct-1 is regulated after incubation with therapeutic agents, including alkylating agent methyl methanesulfonate, etoposide, and cisplatin (60). The results presented here suggest that the Oct-1 activation state may affect endogenous IFN-A expression. Therefore, we suggest that Oct-1 activation should be evaluated in combined therapy associating drugs and IFN-α.

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